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Identification of amino acid residues in HIV-1 reverse transcriptase that are critical for the proteolytic processing of Gag–Pol precursors

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ABSTRACT

The efficient processing of human immunodeficiency virus type 1 Gag–Pol requires not only protease activity but also specific reverse transcriptase (RT) and integrase sequences. However, the critical amino acid residues of the HIV-1 Pol gene involved in protease-mediated Gag–Pol processing have not been precisely defined. Here, we found that the substitution of Thr-128 or Tyr-146 with Ala markedly impaired the proteolytic processing of the MA/CA, p66/p51 and RT/IN sites but did not affect the normal processing of other sites. Moreover, a Thr-128 or Tyr-146 mutation in RT abolished RT dimerization in vitro. These results suggest that Thr-128 and Tyr-146 within the RT region play important roles in protease-mediated Gag–Pol processing.

Structured summary of protein interactions:
RT and **RT** physically interact by cross-linking study (View interaction: 1, 2, 3).
CK2 alpha phosphorylates RT by protein kinase assay (View interaction).

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) Gag–Pol is normally translated by a −1 ribosomal frameshift event occurring at a frequency of approximately 5% during Gag translation, resulting in a 1:20 ratio of synthesized Gag–Pol to Pr55-Gag [1]. The incorporation of Gag–Pol into virus particles requires its interaction with Pr55-Gag [2]. The processing of Gag–Pol is accomplished by viral protease activation during Gag–Pol/Gag–Pol interactions or Gag–Pol multimerization [3]. The proteolytic processing of Gag follows a sequential cascade of events that is kinetically controlled by differential rates of processing at each of the five cleavage sites in Gag [4,5]. Protease-mediated Gag–Pol processing requires the protease as well as domains upstream or downstream of the protease region, such as the reverse transcriptase (RT) and integrase (IN) domains. Mutants with an IN-domain deletion form markedly impaired viral particles due to the impairment of normal protease activity [6]. Mature HIV-1 RT is a heterodimer with two subunits, p66 and p51. The p51 subunit is derived from p66 by the proteolytic removal of the C-terminal RNaseH domain. RT–RT interactions may play important roles in protease activation by promoting Gag–Pol multimerization. RT inhibitors enhance RT dimerization in vitro, resulting in increased protease-specific cleavage of Pr55-Gag and Gag–Pol [7]. However, the deletion of the RT region leads to a marked reduction in protease activation and particle maturation [8]. The critical amino acid residues of the HIV-1 RT that contribute to protease-mediated Gag–Pol processing are not yet known.

In this study, we identified the HIV-1 RT amino acid residues critical for Gag–Pol processing through the analysis of RT phosphorylation. The substitution of Thr-128 and Tyr-146 with Ala severely impaired viral replication, due to defects in proteolytic processing at the MA/CA, p66/p51, and RT/IN sites.

2. Materials and methods

2.1. Plasmids

Details of the plasmid constructs used in this study are provided in the Supplementary materials and methods.

2.2. Virus infection

For infection, 293T cells were co-transfected with 0.1 μg of pMD.G-VSV-G and 0.1 μg of pNL43lucΔenv or pNL43lucΔenv

Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); PR, protease

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containing an RT mutation of interest. At 48 h post-transfection, the culture supernatants were harvested and filtered through 0.45-μm filters. 293T cells were infected with pseudotyped viruses (corresponding to 20 ng of p24). At 24 h postinfection, the cells were harvested and lysed with 300 μl of cell-lysis buffer. An aliquot (10 μl) of each lysate was then subjected to a luciferase assay.

2.3. Preparation of virions

Details are given in the Supplementary materials and methods.

2.4. Preparation of anti-RT sera

Details of anti-RT sera used in this study are given in the Supplementary materials and methods.

2.5. Crosslinking

Crosslinking was performed as previously described [9].

2.6. Phosphorylation assay

Details are given in the Supplementary materials and methods.

3. Results

3.1. Infectivity of RT-mutant viruses

Recent studies have indicated that the phosphorylation of HIV-1 RT might play an important role in HIV-1 replication [10–11]. To evaluate the role of the putatively phosphorylated RT, we predicted the phosphorylation site(s) of RT using the Scansite 2.0 program [12] and generated single-amino-acid Ala substitutions for the Ser, Thr, and Tyr residues that are conserved among HIV-1 and simian immunodeficiency virus strains. We examined the effect of each mutation on viral replication using a single-round infection system (Fig. 1A). In this study, infection with the S156A mutant led to slightly diminished luciferase activity (approximately 75% of the WT level). Similarly, the single amino acid substitutions of Thr to Ala at position 27 (T27A) and Ser to Ala at position 68 or 105 (S68A and S105A, respectively) resulted in lower levels of luciferase activity (60–65% of the WT level). The infectivity of the RT mutant virus possessing a T58A or T107A mutation significantly decreased to 5–10% of the WT-virus level. The introduction of the T131A, T216A, Y318A, T128A, Y146A or Y183A mutations completely abolished viral infectivity.

To evaluate whether HIV-1 RT mutations affect RT activity, we used the Vpr-p66-WT fusion protein (Fig. 1B), which can be efficiently incorporated into virions [13–14]. The infectivity of T58A, T216A and Y318A was partially restored from 27% to 55% of the WT infectivity by complementation with Vpr-p66-WT-V5 (Fig. 1C). In this complementation, Vpr-p66-WT-V5 was efficiently packaged and processed (Fig. 1E). The T107A mutant was not complemented by Vpr-p66-WT-V5. In addition, the level of RNaseH-V5 in T107A was significantly decreased compared with the WT virion, although the Vpr-p66-WT-V5 and p66-WT-V5 levels in the T107A mutant were similar to WT levels. Moreover, the T131A, and Y183A mutations, which resulted in lower levels of p66-WT-V5 and RNaseH-V5, were not complemented despite the presence of Vpr-p66-WT-V5 (1.3–3.5% of the WT level). In the T128A and Y146A mutants, the majority of Vpr-p66-WT-V5 in the virions remained in the form of unprocessed fusion proteins, and these mutants exhibited defective viral replication (0.5–3.1% of the WT level). To further examine whether incorporating Gag–Pol-WT *in trans* restores the infectivity of the mutant viruses, Gag–Pol-complemented viruses were analyzed for infectivity using a luciferase assay (Fig. 1D). With the exception of Y183A, mutant virus infectivity was partially rescued (26–75% of WT level) by this complementation. Of particular note, a Y183A mutation incorporated into the WT Gag–Pol did not complement infectivity. This result was due to the p66-Y183A mutation displaying some inhibitory potency, as indicated by Vpr-p66-Y183A packaging into the WT virion (data not shown). We concluded that the replication defects associated with the T58A, T216A and Y318A mutants were due primarily to the disruption of RT activity, whereas Thr-107, Thr-128, Thr-131, Tyr-146, and Tyr-183 may be involved in Gag–Pol functions in HIV-1 replication.

3.2. Mutations of Thr-128 and Tyr-146 in RT abolish Gag–Pol processing

To study whether the mutant RTs in the viral particles were present in the p66 and p51 form of the RT, we used anti-RT serum to detect virus-associated mutant RTs (Fig. 2A). The T128A, T131A and Y146A mutations were not found in the p51 form of the RT. Although all of the mutant RTs in the viral particles were detected in the p66 form, T128A, T131A, Y146A, and Y318A mutants had slightly reduced levels of the p66 form relative to the WT. These mutants may be subject to PR-mediated degradation [15].

We next examined the profiles of particle-associated viral proteins to identify possible Gag–Pol processing defects in these mutants. The majority of the Gag-related proteins in the T128A and Y146A mutant virions remained as unprocessed Pr55-Gag (Fig. 2B). The defective Gag-processing phenotype of the T128A and Y146A virions was apparent from the altered ratio of the Pr55-Gag and p24 forms of the CA protein (Fig. 2C). Moreover, the protein levels of HIV-1 protease and integrase were significantly reduced in the T128A and Y148A virions (Fig. 2D and E). Thus, a single amino-acid substitution for Thr-128 or Tyr-148 in the RT appeared to be responsible for the observed effects on Gag–Pol processing.

3.3. Proteolytic cleavage of Gag–Pol was severely impaired at the MA/CA and RT/IN sites in T128A and Y146A virions

To determine the defective sites of Gag–Pol processing in detail, we incorporated several truncated Gag or Pol domains into HIV-1 virions *in trans* as fusion partners of Vpr (Fig. S1A). The proportion of the processed products cleaved at the MA/CA and RT/IN sites was significantly reduced in T128A and Y146A mutants (Fig. S1B and F). In contrast, the cleavage patterns for the SP1/NC, NC/SP2, and SP2/P6 sites in T128A and Y146A mutants were similar to those of the WT (Fig. S1C, D and E). These results suggest that protease-mediated Gag–Pol processing in T128A and Y146A virions was partially affected, but these mutants retained protease activity.

3.4. HIV-1 Gag–Pol processing is dependent on RT dimerization

To determine whether RT dimerization was required for Gag–Pol processing, we used DTSSP (3,3′-dithiobis(sulfosuccinimidylpropionate)) to cross-link protein complexes present in the RT-V5-expressing 293T cells. Only RT monomers were detected in the non-cross-linked samples, with the exception of the Y146A mutant. The addition of 0.2 mM DTSSP yielded cross-linked complexes of RT homodimers when RT-WT-V5 was expressed in 293T cells (Fig. 3). A T131A mutation slightly reduced the level of RT homodimers in the presence of DTSSP compared with the wild type. In contrast, an abnormal pattern was observed in the Y146A mutant in the absence or presence of DTSSP, suggesting that Y146A induced improper folding. Moreover, a T128A mutation resulted in very low levels of the dimer under the same experimental conditions. T58A, Y183A and T216A mutations, which resulted in normal Gag–Pol, retained RT dimerization in the presence of DTSSP. These results suggest that

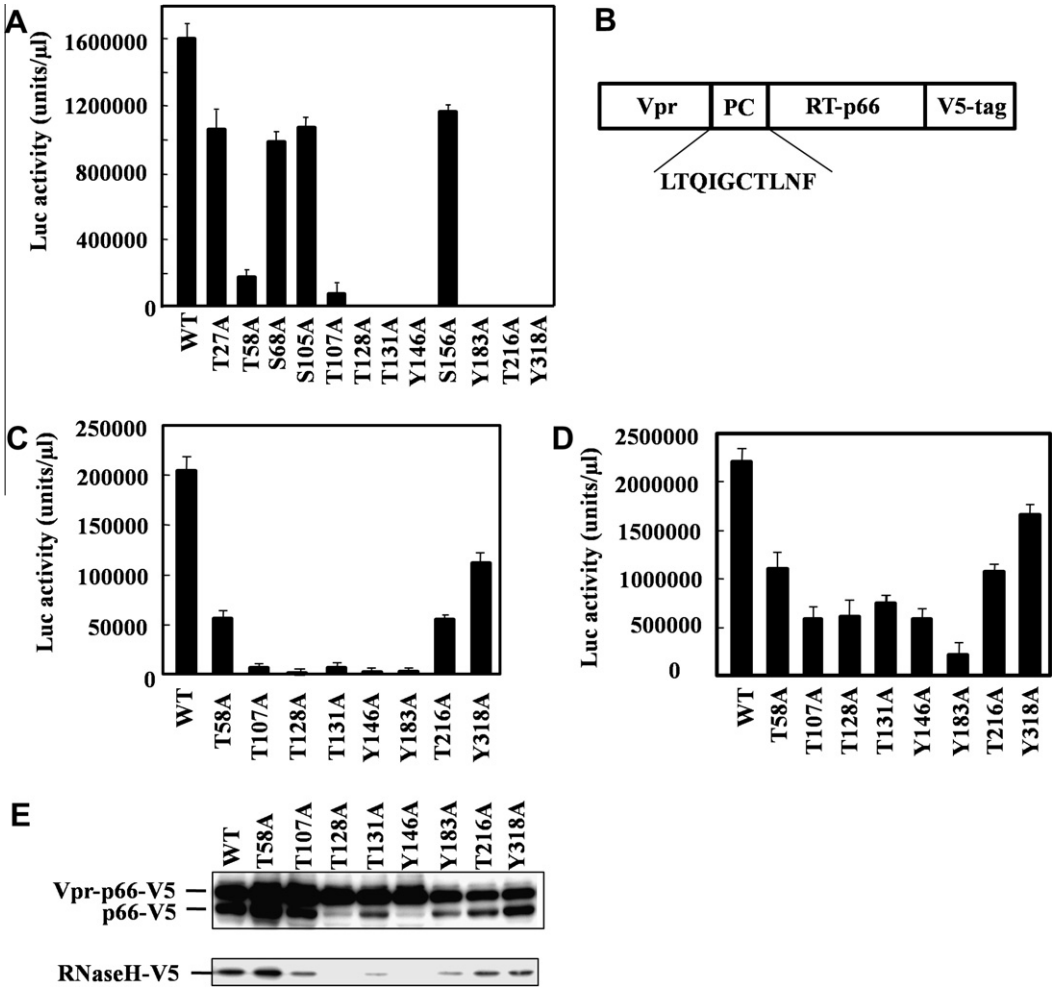

Fig. 1. Infectivity of HIV-1 RT mutants. (A) 293T cells were transfected with 0.1 μg of pMD.G-VSV-G and 0.1 μg of pNL43lucΔenv containing the indicated RT mutations. The 293T cells were infected by pseudoviruses isolated from cell culture supernatants. The luciferase activity in the infected cells was measured 24 h postinfection. (B) An illustration of the Vpr-p66-WT fusion protein including ten amino acids of the protease cleavage sequence at the Vpr-RT junction. (C, D) The 293T cells were transfected with 0.1 μg of wild type (WT) or each RT mutant pNL43lucΔenv, 0.1 μg of pMD.G-VSV-G and 0.2 μg of pVpr-p66-WT-V5 (C) or pMDL-g/p-RRE (D). At 48 h post-transfection, the 293T cells were infected with the indicated pseudotyped viruses. Luciferase activities in the infected cells were measured 24 h postinfection. (E) Culture supernatants in Fig. 1C were collected and subjected to Western immunoblot analysis using an anti-V5 antibody.

RT dimerization may affect Gag–Pol processing. However, the possibility that the T128A and Y146A mutations significantly change the conformation of Gag–Pol precursor, decreasing the accessibility of the targets, cannot be excluded.

3.5. Detection of RT phoshorylation

Harada et al. have reported that HIV RT can be phosphorylated in vitro by casein kinase 2 (CK2-alpha) [16]. We used Phos-tag SDS–PAGE to determine whether CK2-alpha could phosphorylate HIV-1 RT. A phos-tag binds to two Mn^{2+} ions and acts as a phosphate-binding molecule [17]. This complex is used for phosphate affinity SDS–PAGE, in which a mobility shift can be observed with phosphorylated proteins. Recombinant His-RT was incubated with recombinant CK2-alpha in the presence of ATP. As shown in Fig. 4A, a shifted band of recombinant His-RT-WT was observed in the presence of recombinant CK2-alpha in a dose-dependent manner. We next examined the phosphorylation of mutant RT. The phosphorylation levels of His-RT-T128A were similar to wild-type RT (Fig. 4B). In the case of the His-RT-Y146A mutants, the phosphorylation levels were slightly reduced compared with wild-type levels. Moreover, both the T128A and Y146A mutations in recombinant His-RT severely decreased the phosphorylation levels. However, a shifted band corresponding to phosphorylated His-RT-T128A-Y146A could be detected by using a large amount of recombinant His-RT-T128A-Y146A in an in vitro kinase assay (Fig. 4C). To further confirm the phosphorylation of HA-RT-wild and its RT mutants in vivo, HA-tagged RT or its mutants were expressed in 293T cells and the cell lysates were immunoprecipitated with anti-HA antibody. The immune complex was analyzed by Phos-tag, a probe used to detect phosphorylated proteins (Fig. 4D). Wild-type RT was phosphorylated in vivo. However, RT-T128A-Y146A significantly reduced the phosphorylation levels. These results suggest that the T128 and Y146 residues, but not the RT phosphorylation sites, which demonstrated a defect in RT dimerization, affected RT phosphorylation. RT phosphorylation may be required for its dimerization.

4. Discussion

Dunn et al. have reported that an L264S or E302Q mutation in RT rendered the protein susceptible to degradation by PR within virions [15]. In contrast, we identified here the critical amino acid residues (T128 and Y146) that specifically affected RT dimerization but had little effect on the stability of RT. To obtain structural insights into the findings, we constructed a 3-D model of p51/p66 heterodimer of the wild-type (NL43) RT. The model shows that

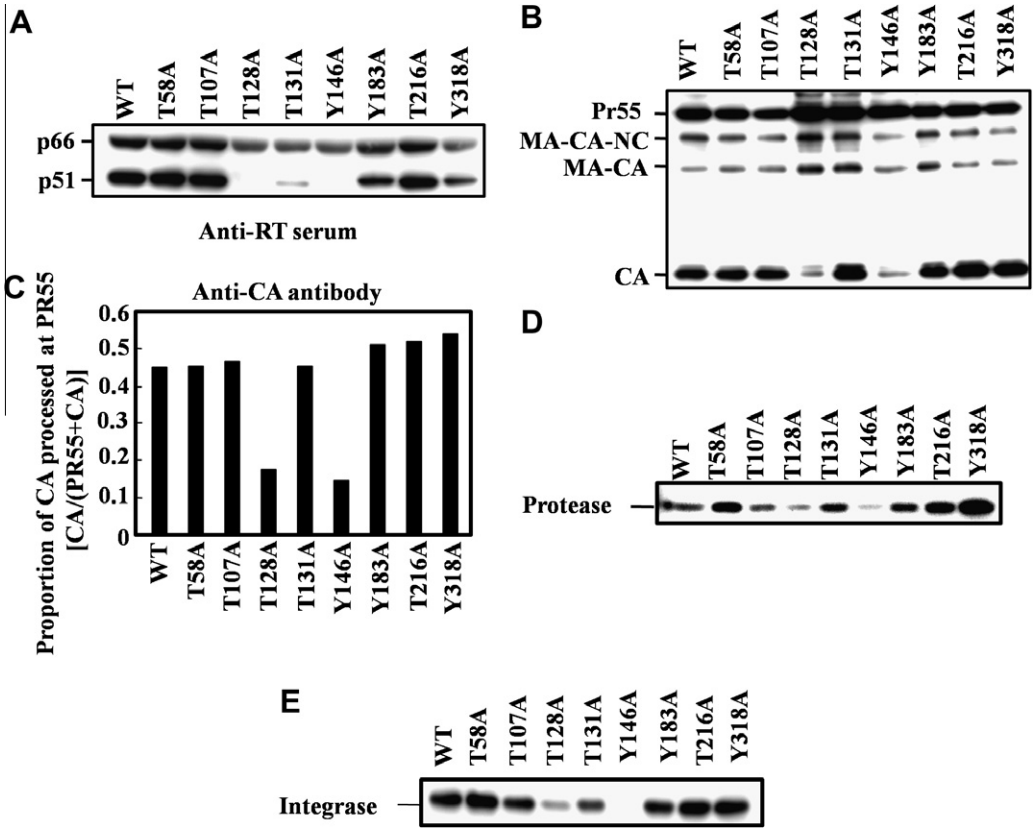

Fig. 2. The effect of RT mutations on the processing of HIV-1 Gag–Pol. 293T cells were transfected with 0.2 μg of each RT mutant pNL4-3. At 48 h post-transfection, the supernatants were harvested and subjected to Western-blot analysis. The Gag–Pol cleavage products were detected using anti-RT sera (A), an anti-CA antibody (B), an anti-protease antibody (D) and an anti-integrase antibody (E). (C) The proportion of CA cleaved at the Pr55-Gag cleavage site was calculated from the integrated band intensities.

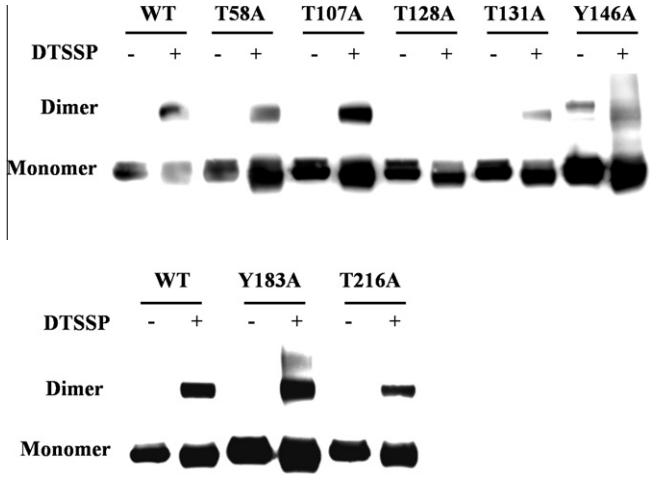

Fig. 3. Evaluation of RT dimerization. 293T cells were transfected with pLenti-p66-V5 or the RT mutants. At 48 h post-transfection, the cells were lysed with CSK buffer containing 0.5% NP-40. The cell lysates were incubated in either the absence or presence of 0.2 mM DTSSP at room temperature for 20 min and analyzed by Western blotting using an anti-V5 antibody.

the L264 and E302 residues are located in the two α-helixes of Thumb domain (Fig. S2A). Because helices are often critical in formation of a stable core structure of a protein, the L264S or E302Q mutations could critically influence proper folding and stability of the Thumb domain. Such effects could affect RT dimerization because the thumb of the p51 subunit extensively interacts with the RNase H domain. Simultaneously, the effects could increase susceptibility of mutants to PR in a virion via exposure of improperly folded domain. In contrast, the T128 and Y146 residues in p51 are positioned at the base of the β7–β8 loop, whose tip is embedded in a small cleft of p66 surface and constituted of a direct interaction surface between p51 and p66 (Fig. S2B). Therefore mutations at T128 and Y146 could influence orientation of the β7–β8 loop and alter stability of the RT dimer. Meanwhile, the mutations would not influence critically the stability of the RT, because the loop is positioned on protein surface. The failure of detection of RT p51 subunit in these mutants (Fig. 2A) may suggest that p66 dimerization is prerequisite for the processing. These structural insights are well consistent with the present experimental findings. However, further studies will be required to clarify the structure–function relationship with regard to the susceptibility of RT to PR degradation.

The mutation of T128 or Y146 in RT impaired the protease-mediated Gag–Pol processing. It is possible that substitutions in RT significantly change the conformation of the Gag–Pol precursor and decrease the accessibility of the targets for the protease. However, the three-dimensional (3-D) model suggests that the T128 and Y146 residues are not positioned near the inherent cleavage sites flanking RT, i.e., N- and C-terminal end of the RT (Fig. S2). In addition, reported electron microscopy studies suggest that Gag precursor has a rodlike structure [18,19], by which the T128 and Y146 residues are probably located far from the cleavage site and other proteins. Therefore, the mutations at the T128 and Y146 are less likely to influence critically the protease accessibility into the inherent cleavage sites of the Gag/Pol precursor protein, although the influences are formally not ruled out at present. An

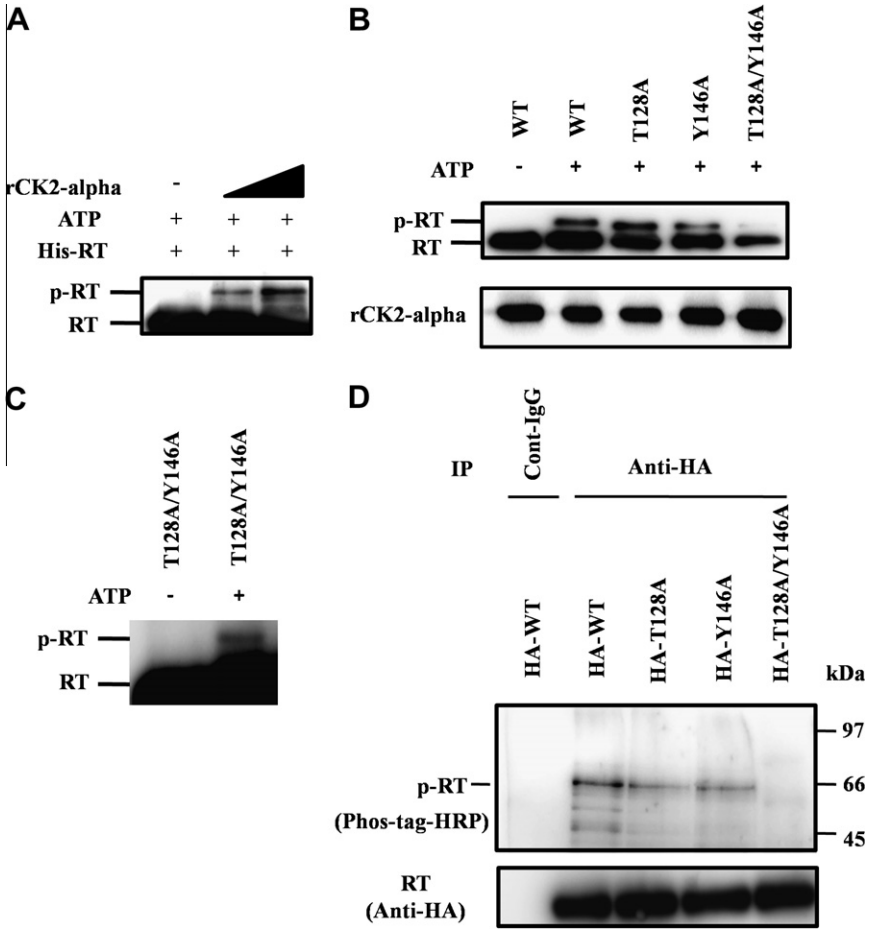

Fig. 4. The phosphorylation of recombinant RT in vitro. (A) Initially, 0.1 μg of recombinant His-RT-WT was incubated with 0.5 mM ATP in the absence or presence of 0.05 or 0.2 μg of recombinant His-CK2-alpha. The phosphorylation of recombinant His-RT was detected using Western immunoblot analysis after Phos-tag SDS–PAGE. The phosphorylation of the recombinant His-RT was indicated as a shifted band. (B, C) 0.1 μg (B) or 1.0 μg (C) of indicated recombinant His-RT was incubated with 0.2 μg of recombinant His-CK2-alpha in the absence or presence of 0.5 mM ATP. The phosphorylation of His-RT was detected as indicated in Fig. 4A. (D) 293T cells were transfected with pcDNA-HA-RT or its RT mutants. At 48 h after transfection, the cells lysates were immunoprecipitated with anti-HA antibody and phosphorylated proteins were detected using Phos-tag-Biotin and Streptavidin-conjugated HRP.

alternative possibility is that the mutations at the T128 and Y146 impaired the protease-mediated Gag–Pol processing via attenuation of Gag/Pol dimerization for PR activation. Further study is necessary to address each of these issues.

These observations are consistent with the hypothesis that antiviral drugs targeting RT might inhibit HIV-1 replication without necessarily inhibiting the catalytic function of the RNA-dependent DNA polymerase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.09.034.

References

[1] Jacks, T., Power, M.D., Masiarz, F.R., Luciw, P.A., Barr, P.J. and Varmus, H.E. (1988) Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. Nature 331, 280–283.
[2] Park, J. and Morrow, C.D. (1992) The nonmyristylated Pr160gag-pol polyprotein of human immunodeficiency virus type 1 interacts with Pr55gag and is incorporated into viruslike particles. J. Virol. 66, 6304–6313.
[3] Louis, J.M., Nashed, N.T., Parris, K.D., Kimmel, A.R. and Jerina, D.M. (1994) Kinetics and mechanism of autoprocessing of human immunodeficiency virus type 1 protease from an analog of the Gag-Pol polyprotein. Proc. Natl. Acad. Sci. USA 91, 7970–7974.
[4] Wiegers, K., Rutter, G., Kottler, H., Tessmer, U., Hohenberg, H. and Krausslich, H.G. (1998) Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. J. Virol. 72, 2846–2854.
[5] Pettit, S.C., Everitt, L.E., Choudhury, S., Dunn, B.M. and Kaplan, A.H. (2004) Initial cleavage of the human immunodeficiency virus type 1 GagPol precursor by its activated protease occurs by an intramolecular mechanism. J. Virol. 78, 8477–8485.
[6] Bukovsky, A. and Gottlinger, H. (1996) Lack of integrase can markedly affect human immunodeficiency virus type 1 particle production in the presence of an active viral protease. J. Virol. 70, 6820–6825.
[7] Tachedjian, G., Moore, K.L., Goff, S.P. and Sluis-Cremer, N. (2005) Efavirenz enhances the proteolytic processing of an HIV-1 pol polyprotein precursor and reverse transcriptase homodimer formation. FEBS Lett. 579, 379–384.
[8] Quillent, C., Borman, A.M., Paulous, S., Dauguet, C. and Clavel, F. (1996) Extensive regions of pol are required for efficient human immunodeficiency virus polyprotein processing and particle maturation. Virology 219, 29–36.
[9] Nishitsuji, H., Hayashi, T., Takahashi, T., Miyano, M., Kannagi, M. and Masuda, T. (2009) Augmentation of reverse transcription by integrase through an

interaction with host factor, SIP1/Gemin2 Is critical for HIV-1 infection. PLoS One 4, e7825.
[10] Davis, A.J., Carr, J.M., Bagley, C.J., Powell, J., Warrilow, D., Harrich, D., Burrell, C.J. and Li, P. (2008) Human immunodeficiency virus type-1 reverse transcriptase exists as post-translationally modified forms in virions and cells. Retrovirology 5, 115.
[11] Idriss, H., Kawa, S., Damuni, Z., Thompson, E.B. and Wilson, S.H. (1999) HIV-1 reverse transcriptase is phosphorylated in vitro and in a cellular system. Int. J. Biochem. Cell Biol. 31, 1443–1452.
[12] Obenauer, J.C., Cantley, L.C. and Yaffe, M.B. (2003) Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res. 31, 3635–3641.
[13] Mulky, A., Sarafianos, S.G., Arnold, E., Wu, X. and Kappes, J.C. (2004) Subunit-specific analysis of the human immunodeficiency virus type 1 reverse transcriptase in vivo. J. Virol. 78, 7089–7096.
[14] Wu, X., Liu, H., Xiao, H., Conway, J.A., Hunter, E. and Kappes, J.C. (1997) Functional RT and IN incorporated into HIV-1 particles independently of the Gag/Pol precursor protein. EMBO J. 16, 5113–5122.
[15] Dunn, L.L., McWilliams, M.J., Das, K., Arnold, E. and Hughes, S.H. (2009) Mutations in the thumb allow human immunodeficiency virus type 1 reverse transcriptase to be cleaved by protease in virions. J. Virol. 83, 12336–12344.
[16] Harada, S., Haneda, E., Maekawa, T., Morikawa, Y., Funayama, S., Nagata, N. and Ohtsuki, K. (1999) Casein kinase II (CK-II)-mediated stimulation of HIV-1 reverse transcriptase activity and characterization of selective inhibitors in vitro. Biol. Pharm. Bull. 22, 1122–1126.
[17] Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K. and Koike, T. (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol. Cell Proteomics 5, 749–757.
[18] Datta, S.A., Curtis, J.E., Ratcliff, W., Clark, P.K., Crist, R.M., Lebowitz, J., Krueger, S. and Rein, A. (2007) Conformation of the HIV-1 Gag protein in solution. J. Mol. Biol. 365, 812–824.
[19] Wright, E.R., Schooler, J.B., Ding, H.J., Kieffer, C., Fillmore, C., Sundquist, W.I. and Jensen, G.J. (2007) Electron cryotomography of immature HIV-1 virions reveals the structure of the CA and SP1 Gag shells. EMBO J. 26, 2218–2226.